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BINDING ASSAYS FOR THE QUANTITATIVE DETECTION OF P. BREVIS POLYETHER NEUROTOXINS IN BIOLOGICAL SAMPLES AND ANTIBODIES AS THERAPEUTIC AIDS FOR POLYETHER MARINE INTOXICATION

ANNUAL REPORT

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1 December 1987

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7001

University of Miami
Coral Gables, Florida 33149-1098

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No 0704-0188 Exp. Date Jun 30, 1986

1a. REPORT SECURITY CLASSIFICATION: Unlassified	1b. RESTRICTIVE MARKINGS: AD-A191 072
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Miami (6b. OFFICE SYMBOL (If applicable))	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Coral Gable, Florida 33149-1098	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research and Devel. Comman (8b. OFFICE SYMBOL (If applicable))	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-87-C-7001
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62770A; PROJECT NO. 3M1 62770A871; TASK NO. AA; WORK UNIT ACCESSION NO. 402

11. TITLE (Include Security Classification)
Binding Assays for the Quantitative Detection of P. brevis Polyether Neurotoxins in Biological Samples and Antibodies as Therapeutic Aids for Polyether ~~Marine Intoxication~~

12. PERSONAL AUTHOR(S): Daniel G. Baden

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 12/1/86 TO 11/30/87	1987 December 1	33

16. SUPPLEMENTARY NOTATION
Cont'd from pg. 1

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	01		Keywords: marine toxins, antibodies, sodium channels, receptor binding, brevetoxin.
06	15		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The polyether lipid-soluble toxins isolated from the marine dinoflagellate Ptychodiscus brevis bind to a unique site, Site V, associated with voltage-dependent sodium channels in rat brain synaptosomes. Using tritiated PbTx-3 as a specific probe for binding as Site V, a Kd of 2.9 nM and a Bmax of 6.8 pmoles/mg protein has been determined. Binding equilibria and displacement by unlabeled PbTx-3 occur in a comparable concentration range to that of saxitoxin site I). Labeled toxin can be displaced in a competitive manner by any of the other 5 naturally-occurring toxins; the quantitative displacement ability of each appears to reflect individual potency in fish bioassay. Preliminary Ki calculations have been made for four of the toxins. A comparison of ED50 in radioimmunoassay and ED50 in synaptosome binding assay indicates that the former assay is useful for detection of toxins which possess structural backbone of PbTx-3, the immunizing hapten. Thus the two assays have quantitative applicability; the former with respect to potency and the latter with respect to

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☑ SAME AS RPT ☐ DTIC USERS	unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Mary Frances Bostian	301-663-7325	SGRD-RMI-S

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

Block 19 continued

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Summary

The polyether lipid-soluble toxins isolated from the marine dinoflagellate Ptychodiscus brevis (formerly Gymnodinium breve bind to a unique site, Site V, associated with voltage-dependent sodium channels in rat brain synaptosomes. Using tritiated PbTx-3 as a specific probe for binding at Site V, a K_d of 2.9 nM and a B_{max} of 6.8 pmoles/mg synaptosomal protein has been determined. Binding equilibria and displacement by unlabeled PbTx-3 occur in a comparable concentration range to that of saxitoxin (site I). Labeled toxin can be displaced in a competitive manner by any of the other 5 naturally-occurring toxins; the quantitative displacement ability of each appears to reflect individual potency in fish bioassay. Preliminary K_i calculations have been made for four of the toxins. A comparison of ED_{50} in radioimmunoassay and ED_{50} in synaptosome binding assay indicates that the former assay is useful for detection of toxins which possess the structural backbone of PbTx-3, the immunizing hapten. Thus, the two assays have quantitative applicability; the former with respect to potency and the latter with respect to structure. Preliminary experiments involving conversion of the radioimmunoassay to a urease enzyme linked form have been successful.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) haved adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, revised 1985).

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II. Statement of the Problem

This contract is concerned with the development of diagnostic methods and therapy for exposure to polyether toxins produced by marine dinoflagellates. We have proposed two separate binding assays which have potential value in the quantitative detection of these toxins in biological samples. Our specific aims are to:

(1) develop and refine *in vitro* radiometric binding assays to detect polyether marine neurotoxins in biological samples using tritiated brevetoxin PbTx-3 (formerly T17) as radiometric probe and employing:

(a) antibodies prepared in a goat against toxic component PbTx-3 produced by laboratory culture of *Ptychodiscus brevis*;

(b) synaptosomes from rat brain;

(2) determine the sensitivity and specificity of the binding assays using brevetoxin standards mixed with biological samples of clinically-obtainable types, i.e.serum, mucousal secretions, urine and or feces;

(3) using goat antibodies or solubilized brevetoxin binding component from rat brain, develop enzyme-linked assays to further simplify the procedure for routine use;

(4) examine potential cross-reactivity of the binding assays with respect to other polyether toxins, and hence their usefulness in the detectioon of other lipid-soluble marine polyether toxins;

(5) examine the feasibility of using available antibodies as therapeutic agents, first using competitive *in vitro* molecular pharmacological binding assays, and later by examining the reversal of toxic effects in animals by immunoassay;

(6) provide reagents adequate for 10,000 assays, including radioactive toxin probe, and data on tests and evaluations. Detailed protocols will accompany reagents.

III. Background

A. History

At the time of contract submission, we reproducibly were producing two toxins, PbTx-2 (formerly T34) and PbTx-3 (formerly T17), in mg amounts from laboratory cultures of Florida's red tide dinoflagellate *Ptychodiscus brevis* (1) We now routinely purify to homogeneity six brevetoxins, based on two structural backbones (Figure 1) (2). All toxins produced by *P. brevis* are ichthyotoxins, and in fact most investigators utilize fish bioassay to precisely identify potent fractions during purification. This complex of six toxins, namely PbTx-1,-2,-3,-5,-6, and -7, in composite are responsible for *in situ* fish kills during red tides in the Gulf of Mexico (3). In addition, these polyether materials are acutely potent in swiss white mice (4,5), as bronchoconstrictors (6), in *in vitro* phrenic nerve

hemidiaphragm preparations (7), and in crayfish and squid giant axon (8). In varying degrees, they also elicit increased sodium ion influx in brain synaptosomes (9), and induce increased release of acetylcholine and decreased choline uptake in neuromuscular junction (3). Dose-response curves for each effect enumerated above were in the nM to pM concentration ranges. In all *in vitro* systems, each described effect was reversible by washing with fresh toxin-free bathing medium.

Squid giant axon and crayfish axon bundle experiments were particularly important in our initial work (8), for these gave us our first indication that brevetoxins interacted with specific sites of the axons. With a tetrodotoxin-sensitive mechanism of action, brevetoxins induced a sodium ion-sensitive dose-dependent depolarization (30 mV maximum depolarization, linear dose-response between 0.2-100 nM PbTx-3). This data led us to postulate a specific binding site for the brevetoxins located on, or proximal to, the voltage-sensitive sodium channel (9).

B. Toxins

Since the identification of *P. brevis* as the progenitor of Florida red tide toxins, the potent entities have been know by several different names--most of which are important only for historical reasons. The name brevetoxins seems to have been adopted, but a widely used notation series has not been adopted. Following conventional notation for natural toxins (which is currently under review by several National and International working groups), we have adopted the notation PbTx-# for *P*tychodiscus *b*revis *T*ox*i*n 1-8 (Table 1). Additional toxins, if discovered, would continue with PbTx-9 etc. It is expected that, like the PbTx-2 backbone (Type 1), both epoxides and O-acetates of PbTx-1 (Type 2) will ultimately be discovered. The mass amounts of these materials is expected to be approximately as indicated in Table 2.

TABLE 1. NOMENCLATURE FOR THE BREVETOXINS*

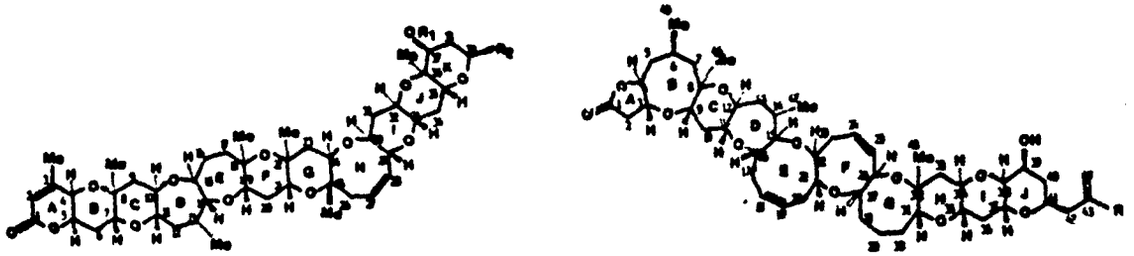
Notation	Synonyms	Reference
PbTx-1	Brevetoxin-A	10,11
	GB-1	11,12
PbTx-2	Brevetoxin-B	13
	GB-2	11
	T34	4
PbTx-3	GB-3	11
	T17	5
PbTx-4	GB-4	11,14
PbTx-5	GB-5	11
PbTx-6	GB-6	11
PbTx-7	GB-7	11
PbTx-8	Brevetoxin-C	15

*refer to Figure 1 for structure details

TABLE 2. TOXIN YIELDS FROM CULTURES
TYPE 1 VERSUS TYPE 2 TOXINS

Type 1 Notation	Yield (pg/cell)	Type 2 Notation	Yield (pg/cell)	Note
PbTx-2	8.7	PbTx-1	1.7	(1)
PbTx-3	0.42	PbTx-7	0.026	(2)
PbTx-5	0.062	PbTx-9*	0.013	(3)
PbTx-6	0.037	PbTx-10*	0.008	(4)

*Toxins underlined have not been demonstrated in cultures. By analogy with Type 1 toxins, they are proposed to exist, and the yields given are in proportional amounts to Type 1 analogies. Notes: (1) alpha-beta unstaurated aldehyde; (2) alpha-beta unsaturated primary alcohol; (3) O-acetate; (4) epoxide.



	R_1	R_2
PbTx-2	H	$CH_2C(=CH_2)CHO$
PbTx-3	H	$CH_2C(=CH_2)CH_2OH$
PbTx-5	Ac	$CH_2C(=CH_2)CHO$
PbTx-6	H	$CH_2C(=CH_2)CHO$ (27,28 epoxide)
PbTx-8	H	CH_2COCH_2Cl

	R
PbTx-1	CHO
PbTx-7	CH_2OH

No structural information available on PbTx-4

Figure 1. Structures of the Brevetoxins.

C. Molecular Pharmacology

Initial binding experiments performed by others were indirect in nature, with respect to brevetoxin binding; i.e. unlabeled brevetoxin was used as potential competitor for other toxins known to bind to specific sites associated with voltage-sensitive sodium channels. Using this type of protocol, it was illustrated that PbTx-1 did not displace toxins which bind specifically at sites 1-4 located on, or proximal to, the channel (16,17).

In vivo, binding of brevetoxin to site 5 of voltage-sensitive sodium channels is believed to be the pharmacologically-significant event in the onset of intoxication (9,17). Using tritiated brevetoxin PbTx-3 (C-42 tritium covalent label) as the specific probe, binding was determined at 4°C in rat brain synaptosomes using a rapid centrifugation technique (9). Rosenthal analysis yields a K_D of 2.9 nM and a B_{max} of 6.8 pmol of toxin/mg of protein (Fig 2). Labeled probe can be displaced by unlabeled PbTx-3, PbTx-2, or synthetic PbTx-3 (reduced PbTx-2) but not by a nontoxic, synthetic oxidized derivative of PbTx-2 (9). Competition experiments using unlabeled natural toxin probes specific for sites 1-4 of the voltage dependent sodium channel illustrated that indeed the tritiated brevetoxin binds to a previously-undescribed site (9).

Using naturally-occurring and derivative brevetoxins, we began a quantitative study of brevetoxin binding in this assay. Specific binding assays using sodium channel receptors reflect potency of the individual toxins in fish bioassays.

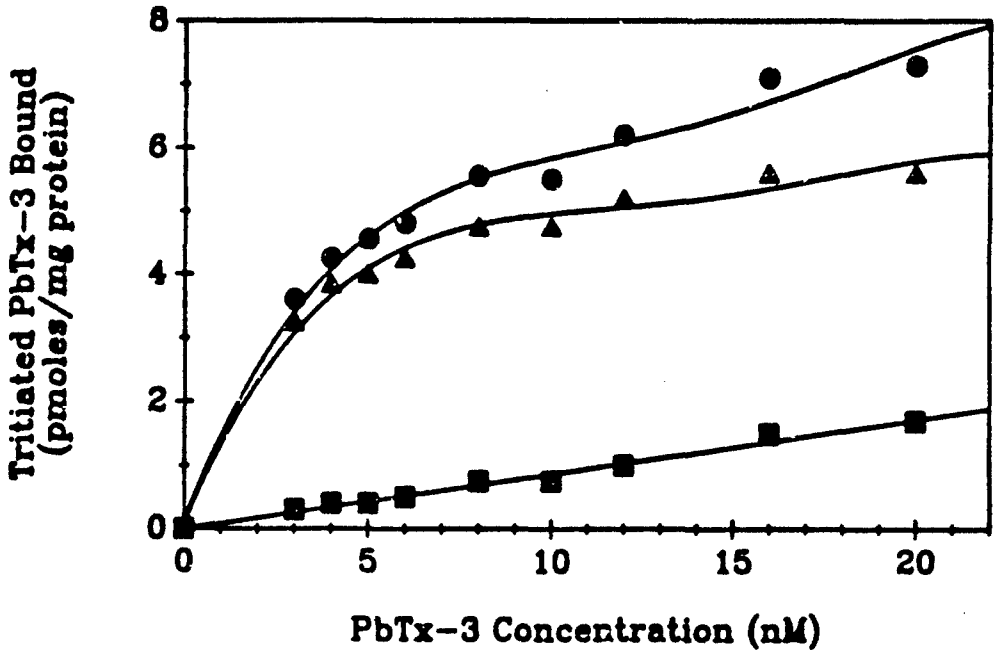


Figure 2. Concentration dependence of Tritiated PbTx-3 Binding to Rat Brain Synaptosomes. (●) Total binding, (▲) specific binding, (■) nonspecific binding [in presence of 10 uM unlabeled PbTx-3]. 4°C, 1 hour incubation.

D. Immunology

At a time when only the structures of PbTx-2 and PbTx-3 were known, we began developing immunoassays for the detection of brevetoxins in marine food sources (18). Utilizing bovine serum albumin-linked brevetoxin PbTx-3 as complete antigen, we succeeded in producing antiserum in a goat. We chose goats for the large quantities of immune serum which we could obtain, provided we could raise an antibody population. Subsequent characterization of the immune serum obtained indicated that both PbTx-2 and PbTx-3 were detected in approximately equivalent manners. Although oxidized PbTx-2 was not potent in either fish or mouse bioassay (9), it did displace PbTx-3 in competitive radioimmunoassay, an indication that potency was not reflected in RIA.

With the description of new brevetoxins based on the PbTx-2,PbTx-3 -type structural backbone (11), it was of interest to examine the competitive abilities of these new toxins. Based on the types of structural derivatives in tthis toxin series, we felt that new information regarding the epitopic sites on the brevetoxin backbone might be uncovered. In the same vein, the new structural backbone present in PbTx-1 and PbTx-7 might give us further insight into epitopic sites (the terminal 3 to 4 rings are identical) on brevetoxins.

In addition, we began to explore methods for converting the RIA to an enzyme linked form. We sought to use an enzyme system which (1) was stable, (2) produced a color reaction which would be visible to the naked eye (even though our evaluation would take place in a microtitre plate reader), (3) would lend itself to coupling enzyme to either toxin or antibody, and (4) would possess an enzymatic activity that was absent in mammalian systems (to reduce background color reactions).

IV. Technical Approach

A. Dinoflagellate Culture

Unialgal cultures of Ptychodiscus brevis were grown in the laboratory in 10 liter batches in glass carboys. Carboys were kept in continuous light at 4000 lux and 24°C in plexiglass constant temperature baths. Typically, 80 liters of culture are harvested each week, 650 liters of culture being grown continuously. Cultures at peak density, if not extracted, were diluted 50/50 with autoclaved NH-15 medium (19). Twenty-one days after inoculation, cultures reach maximum density. Cultures were harvested in either mid-logarithmic phase or in stationary phase, for reasons described in V. A. Toxins.

B. Toxin Purification

Toxin was extracted from whole 10 liter cultures by the addition of 1 liter of chloroform. Cells were disrupted and cultures mixed by use of a vibrating liquid homogenizer. Following flash-evaporation of the chloroform fraction, the residue was dissolved in 90% aqueous methanol and was extracted three times with equal portions of petroleum ether to remove nontoxic pigments. The residue which remained after evaporation of the methanol fraction was extracted with acetone and non-toxic insoluble materials were removed by centrifugation. The semipurified toxins were next subjected to three silica gel chromatographic procedures. The first silica gel step, a flash column, was performed using $CHCl_3$/methanol/acetic acid (100/10/1) as developing solvent. Two column volumes of solvent were passed through the column, collecting all eluent for flash-evaporation. This step is necessary prior to thin-layer chromatography.

A preparative thin-layer chromatography step using silica gel plates (1000 u thickness) utilizes acetone/petroleum ether (30/70) as solvent and resolved two toxic fractions (each of which is composed of multiple individual toxins)(R_f= 0.17 and 0.34). A second thin-layer chromatography step (500 u thickness) utilizes ethyl acetate/petroleum ether (50/50) for R_f= 0.34 material; a similar step utilizing a solvent system of 70/30 ethyl acetate/petroleum ether. The individual toxins can be visualized under short wave ultraviolet light. Toxins were eluted from the silica gel using acetone or methanol. TLC purified toxins were subjected to C-18 reverse phase high pressure liquid chromatography (1.4 mL/min, 85% methanol/15% water, isocratic, uv detection at 215 nm). The entire purification procedure requires two days; the first day progresses through both thin-layer steps (a substantial increase in efficiency over that outlined in year 1) and the second day is required for HPLC.

C. Synaptosome Binding Assay

Biological Preparation. Synaptosomes were prepared fresh daily from rat brain using the techniques described by Dodd et al. (20). Synaptosome integrity was evaluated using electron microscopy, or by ^{22}Na influx experiments. To prepare lysed synaptosomal fragments, the synaptosomal pellet was resuspended in 5 mM sodium phosphate (pH 7.4) and incubated with occasional stirring for 30 min in an ice bath. Protein was measured on resuspended intact synaptosomes or lysed synaptosomes just prior to binding experiments using the technique described by Bradford (21).

Toxin probe preparation. Natural toxins were used as obtained, purifed from cultures. Synthetic tritiated PbTx-3 and unlabeled PbTx-3 were prepared by chemical reduction of PbTx-2 using sodium borotritiide or sodium borohydride, respectively. Toxin PbTx-7 was produced by identical chemical reduction of PbTx-1 using borohydride. Precursor toxins were mixed with equimolar reductant, each present in saturated solution. Under stirring conditions, the reactants were mixed and allowed to react for 3.5 min, after which time excess acetone was added as sacrificial substrate (reduced to propanol). The solvent and propanol was evaporated, and the residue was redissolved in minimal acetone. Acetone-soluble material was thin-layer chromatographed on silica gel plates using ethyl acetate/petroleum ether 70/30 as solvent, followed by high pressure liquid chromatography using an isocratic elution (1.4 mL/min) solvent of 85% methanol/15% water and monitoring absorbance at 215 nm.

Tritiated toxin was quantified employing uv HPLC detector tracings and standard curves were developed using unlabeled toxin PbTx-3. Radioactivity was determined using liquid scintillation techniques and appropriate quenched tritium standards. HPLC-purified radioactive PbTx-3 has a specific activity of 10-15 Ci.mmole, or one-fourth the specific activity of the chemical reductant. Aliquots of tritiated toxin are stored under nitrogen atmosphere at -20°C in theyl alcohol solution. Labeled toxin is stable for 4-6 months, repurification by HPLC being performed as necessary.

Other toxins. Other brevetoxins were used as purified from cultures. Potency of individual brevetoxins was measured using Gambusia fish bioassay (2,5).

Binding assays. Binding of tritiated toxin was measured using a rapid centrifugation technique (9). Binding assays were performed in a binding medium consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, 1 mg/mL bovine serum albumin, and 0.01% Emulphor EL-620 as an emulsifier; the latter being necessary to solubilize the high concentration of unlabeled PbTx-3 used in measurement of nonspecific binding. Binding experiments were also conducted in a depolarizing medium consisting of 135 mM KCl, 5.5 mM glucose, 0.8 mM magnesium sulfate, 1 mg/mL bovine serum albumin in 50 mM HEPES (pH 7.4). Synaptosomes (40-80 ug

total protein), suspended in 0.1 mL binding medium minus BSA were added to a reaction mixture containing tritiated PbTx-3 and other effectors in 0.9 mL binding medium in 1.5 mL polypropylene microcentrifuge tubes. After mixing and incubating at 4°C for 1 hour, samples were centrifuged at 15000 g for 2 minutes. Supernatant solutions were aspirated and the pellets were rapidly washed with several drops of a wash medium (9). The pellets were then transferred to liquid scintillation minivials containing 3 mL scintillant and the bound radioactivity was estimated using liquid scintillation techniques. Nonspecific binding was measured in the presence of a saturating concentration of unlabeled PbTx-3 (10 uM) and was subtracted from total binding to yield specific binding. Free tritiated probe was determined by counting directly an aliquot of the supernatant solutions prior to aspiration.

D. Immunoassay

Antigen Construction. Toxins PbTx-2 and PbTx-1 were purified to HPLC homogeneity as described above, and potency was confirmed in each case using the Gambusia affinis bioassay (2,5). The toxins were unlikely to be antigenic because of their molecular size, and thus it was necessary to couple them as haptens to a suitable antigenic carrier, in these cases to bovine serum albumin (BSA). We utilized the aliphatic aldehyde functions present in each PbTx-2 and PbTx-1 as the coupling site, principally because the aldehyde in each case is located on the terminal portion of each molecule and thus the toxin's spatial exposure during immunization would be enhanced.

Homogeneous toxin, either PbTx-2 or PbTx-1, was added to acetonitrile to yield a final concentration of 7 mg/mL. To this solution was added (in 3 equal increments at one minute intervals) sodium borohydride (as a saturated solution in acetonitrile). The final acetonitrile stoichiometry added was on the order of one Mole reducing equivalents per Mole toxin. For assessment of reducing efficiency, and as a tracer for later coupling steps, one uCi tritium labeled borohydride was added to the reaction mixture. Following reaction for 6 minutes under conditions of constant stirring, excess borohydride was degraded by the addition of one mL acetone (which is reduced to propanol). The resulting solution was thin-layer chromatographed on silica gel as described on page 11, and ultimately was purified to homogeneity using HPLC. For purposes of our later coupling, we sought to utilize only "peak 1" (see results and Discussion) reduction product. However, we wish to point out that "peak 2", which corresponds to a doubly reduced product, also possesses a primary alcohol and could be used for coupling as well.

Purified PbTx-3 (reduced PbTx-2) or PbTx-7 (reduced PbTx-1) was dissolved in a minimal volume of redistilled pyridine, and a ten-fold molar excess of succinic anhydride in pyridine

was added with stirring. The reaction vial was sealed, and was heated to 85°C and stirred for 2 hours in an oil bath . Following reaction, each solution was dried under a stream of nitrogen, redissolved in minimal methanol, and was chromatographed in 70/30 ethyl acetate/ petroleum ether as described on page 11. Portions of each plate were sprayed with bromcresol green solution for detection of acids, and 1 cm portions of each plate were scraped and assayed for radioactivity by liquid scintillation techniques. Fractions which produced both acid-positive reaction (succinic acid) and radioactivity (toxin) were scraped, eluted, and weighed.

The free carboxyl function on each toxin-succinate derivative was covalently coupled to the epsilon-amino group of lysine resides in BSA by use of standard techniques we have employed previously (18). The procedure used was that employed for the covalent modification of steroid hormone when coupled to protein carriers (22), except that the final condensation step was lengthened to 12 hours. Following coupling, the mixture was dialyzed against 0.1 M sodium phosphate buffer (pH 7.4) for 24 hours, and 12 hours against phosphate-buffered saline (PBS). Antigen concentration was adjusted to 1 mg/ml toxin equivalents for immunization.

Immunization. A single female goat was immunized with 0.75-1.0 mg toxin equivalents of complete antigen at 2 week intervals for eight weeks, the first immunization containing complete Freund's adjuvant, and subsequent boosts being suspended in incomplete Freund's adjuvant. Thereafter, boosting proceeded at 21-day intervals. Serum was obtained just prior to the third immunization (six weeks), and thereafter just prior to each boost, for evaluation of serum titre. After the ninth immunization (approximately 20 weeks), two additional animals were introduced into the immunization protocol. These animals were pre-bled for baseline serum titres, and then were immunized as described above. These latter two animals are currently being maintained; the first animal was killed and bled, for reasons described in the Results section.

Preparation of Antibodies. Each antiserum was treated with ammonium sulfate to yield a final salt concentration of 1.9 M. The mixture was stirred at 4°C for 1 hour, and was then centrifuged at 12,000 x g at 4°C for 30 minutes. Precipitates were washed once with 1.9 M ammonium sulfate, dissolved in PBS, and dialyzed against PBS for 24 hours. The resulting crude antibody solutions were adjusted to 25 mg/ml protein for titre evaluation. Protein concentrations were measured according to the method of Bradford (21).

Evaluation of Titers in Serum Samples. For each bleeding, 30 uL aliquots of antibody preparation (0.75 mg protein) were added to duplicate tubes containing 0.5 mL PBS, and increasing amounts of [^{3}H]PbTx-3 ranging from 2.0 to 2000 pg. A parallel null experiment was performed using pre-immune serum fractions, for evaluation of specific yet not brevetoxin antibody-specific binding. Non-specific binding in each case was determined in the presence of 10 uM

unlabeled PbTx-3. As in the case with molecular pharmacological binding experiments (see figure 2), specific binding is defined as the difference between total and non-specific binding, i.e. it is a calculated value. The difference between specific binding values in pre- and post-immunization sera is defined as a measure of specific antibody induction. Ideally, pre-immune serum should exhibit only non-specific binding of tritiated brevetoxin. Incubation times and assay protocol are given below.

Radioimmunoassay. The procedure utilized was that described by Bigazzi et al. (23) for the quantitation of serum digoxigenin levels in plasma. Aliquots of antibody solution (0.75-1.25 mg protein) were added to duplicate tubes containing 0.5 mL PBS, 2 ng [^{3}H]PbTx-3, and known quantities of unlabeled toxin ranging from 0.6 to 200 ng. Duplicate control tubes were included, but without unlabeled toxin (total counts bound by antibody), or in the absence of antibody (total counts per tube). The incubation volumes were kept constant by the addition of PBS where needed.

Specific Assay Protocol. After incubation at 24°C for one hour and then overnight in the refrigerator, 0.5 ml of a suspension containing 1% charcoal (RIA grade) and 0.25% dextran in PBS was then added to each incubation tube except for those containing no antibody. Tubes were then mixed and incubated at 4°C for five minutes, and then were centrifuged at 1000 x g for 8 minutes. Aliquots of 0.5 ml were placed in liquid scintillation mini-vials together with 3 ml liquid scintillant and counted against quenched standards in a Beckman LSC with an efficiency for tritium of 58%. Samples were counted for sufficient time to yield counting precision of 95%.

Development of Enzyme-Immunoassay. All assays were carried out in flat bottom 96-well polystyrene microtitre plates (Costar). Each well has a 0.3 mL capacity and a 6.4 mm well diameter. Our work this year has been concerned with development of protein A-linked enzyme, but we have plans for toxin-enzyme and antibody-enzyme work next year. The assay we are seeking to develop is a Protein-A-urease linked sandwich assay. We chose this for the following reasons: (1) protein A binds specifically to the Fc region of IgG and thus will minimize interference with Fab-toxin interactions; (2) protein-A interacts with most IgG Fc's, thus permitting its use for many antibodies created; (3) protein-A-urease is available commercially, thus assuring quality control; (4) urease has a high turn-over substrate rate and is not typically a mammalian enzyme, thus providing low background when examining biological fluids; (5) the assay is conveniently monitored using a dye-coupled (590 nm detection) reaction in response to released ammonium ion, allowing for microtitre plate monitoring colorimetrically.

The assay under development utilizes initial binding of hydrophobic toxin to polystyrene (virtually quantitative), followed by specific antibody binding, followed by washing. Protein A-urease is next added to bind specifically any toxin-

specific antibody bound to adsorbed toxin. After a final wash, urea substrate solution containing bromcresol purple dye is added and the color reaction is evaluated photometrically.

PbTx-2 was used as hapten bound to the plate because of its higher hydrophobicity. Parameters such as optimal toxin concentration per well (in 200 uL PBS), optimal buffering solution for toxin adsorption, and optimum time and temperature for binding. Following toxin binding, brevetoxin specific antibody was added and parameters of time and temperature for binding, antibody concentration, and nonspecific protein blocking or lack of blocking were evaluated. Both of these perturbations were evaluated by classical "chequer board" arrays in the microtitre plates. Evaluation of stability of the microtitre plate-toxin-IgG complex in lyophilized state was evaluated for shelf-life.

The final protocols used were: (1) stock PbTx-2 concentrations in PBS (pH 7.4) of 5 ug/mL, 200 uL per well (one microgram per well) for 2 hours at 37°C, followed by 22 hours at room temperature. Remaining toxin was removed by aspiration. (2) Two hundred uL of 10% BSA in PBS was added to each well, incubated for 24 hours to block nonspecific protein binding sites. (3) Aspiration of BSA solution was followed by addition of antibody solution in 200 uL PBS (approximately 0.5 mg protein) and incubation for 2 hours at elevated temperature and 22 hours at room temperature, as described for toxin above. (4) Excess antibody was removed by aspiration and was retained to be used again. (5) Protein-A urease conjugate (Allelix; Boehringer-Mannheim) was added, 100 uL per well, at a working concentration of 100 uL stock conjugate to 4.9 mL PBS, and the plate was incubated for 0.5 hour at 37°C. (6) Conjugate was aspirated, each well was washed briefly with PBS buffer, followed by a brief distilled water wash. This step is very important to remove buffer which would interfere with the color reaction (as it is a colorimetric pH indicator. (7) Urease substrate (Allelix; Boehringer-Mannheim) in 100 uL aliquots was added to each well. The substrate contains urea and bromocresol purple, which provides a color reaction changing from yellow to deep violet. (8) Absorbance is monitored ideally at 590 nm; our closest filter is 595 nm.

V. Results and Discussion

A. Toxins

Number. We routinely isolate six brevetoxins from laboratory cultures of P. brevis, all based on the two polyether backbones (11). In logarithmic cells, the two predominant toxins are PbTx-1 and PbTx-2 (see Figure 1). In staticnary cells, approximately the same relative amounts of PbTx-1 and PbTx-2 are present on a per cell basis, but now in addition PbTx-3, PbTx-5, PbTx-6 (based on the backbone present in PbTx-2), and PbTx-7 (based on the backbone present in PbTx-1) appear.

Abundance. In logarithmic phase cells, the respective yields of PbTx-1 and PbTx-2 are: 1.7 and 8.7 pg/cell (based on 80 liter extractions, n=10). In stationary cells, none of the "new" toxins exceed 5% of the total mass of toxin present (based on 80 liter extractions, n=3)(refer back to Table 2 for specific yields).

Effects of Culture Conditions. As we outlined in last year's annual report, it appears that culture stage plays an important role in the multiplicity of brevetoxins present. Our results, based intuitively on roughly 2400 liters of extracted culture (taken 80 liters at a time), and empirically on 1040 liters of extract, indicate that logarithmic cells contain primarily the two alpha-beta unsaturated aldehyde toxins; while stationary cells contain the multiplicity of toxins. We had originally thought that perhaps Ca^{2+} ions concentration played a role in toxin profile (based on a mistaken recipe for MH-15 medium preparation which was carried for six months), but subsequent examination of toxin production with respect to Ca^{2+} concentration (restoring concentrations to their higher value) indicates no shift. That we observe a greater number of toxins than we did when cultures were at the Medical School facility is unexplained, and we believe there is no way to examine the difference.

Hypothesis of Toxin Synthesis. Consistent with our observations on toxin profile and culture growth phase is the hypothesis that the toxins extracted from P. brevis may not be synthesized by the dinoflagellate as a normal cellular metabolite. This is, we feel, a rather revolutionary statement, that P. brevis does not synthesis toxin as a metabolic strategy or for any competitive advantage.

Consider for a moment that, as an example, a plastid or plasmid were responsible for the critical "metabolic machinery" necessary to produce brevetoxin from normal cellular substituents. Further consider that the toxins are in themselves deleterious to the dinoflagellates. Assuming that toxin synthesis is constituitive and not an inducible phenomenon, it is possible that a sublethal toxin concentration per cell can be maintained by vegetative binary fission as occurs during logarithmic phase. Upon changes in growth characteristics of cultures (as occurs in stationary

growth phase), however, that delicate balance is upset in favor of toxin synthesis.

Aside from excretion of the two aldehyde toxins PbTx-1 and PbTx-2 (which have been shown by a number of investigators to be intracellular in log phase), there are relatively few ways to detoxify these materials. We postulate that the dinoflagellate detoxifies the two alpha-beta unsaturated brevetoxins to the other toxins within the profile. This is done by normal detoxification reactions including aldehyde reduction (to PbTx-3 and PbTx-7), epoxidation (to PbTx-6), and O-acetate derivatization of the C-37 alcohol in PbTx-2 (to PbTx-5).

Not only is this idea plausible to invoke for P. brevis toxin profiles, but the idea is also consistent with Gonyaulax toxin profiles; i.e. that saxitoxin is synthesized using "machinery" which is partly plastid or plasmid encoded. The derivatives which result and which contribute to profile multiplicity include neosaxitoxin (N-hydroxylation), gonyautoxins 1-4 (sulfate derivatives of hydroxyls), gonyautoxins 5-6 (carbamoyl-N-sulfated derivatives), and epigonyautoxin 8 and gonyautoxin 8 and C3 and C4 (which are combinations of two detoxification pathways).

A step further removed is the situation with ciguatoxin production by Gambierdiscus toxicus, a dinoflagellate which produces ciguatoxin in the wild, but under controlled laboratory conditions does not. Perhaps G. toxicus does not cease ciguatoxin production in the lab, but rather merely becomes more efficient at "detoxifying" the potent material.

We believe it is important to note that in all cases the derivatized materials are less potent than are the parent molecules; and are also more water soluble in a general sense. Not only does this change in intuitive point of view shed light on the toxin-dinoflagellate relationship and the dinoflagellate's capability for dealing with these materials, but it also provides potential for further work on transfer of toxin synthetic capability.

B. Quantitative Assays

Competitive Displacement of Tritiated PbTx-3 by Natural Brevetoxins in Sodium Channel Assays. We have previously shown that PbTx-3 binds to site 5 associated with voltage-sensitive sodium channels, have determined a K_D of 2.9 nM and a B_{max} of approximately 7 picomoles/mg synaptosomal protein (see Figure 2). We also demonstrated in our last annual report that tritiated PbTx-3 could be displaced in a specific manner from its binding site by PbTx-2, or PbTx-3 (either natural or synthetic), but not by oxidized PbTx-2. Our initial observation was that displacement efficiency was linked in a positive fashion with potency in animals.

The sensitivity and specificity of the synaptosomal assay for site 5 using brevetoxin PbTx-3 is equivalent to the case for synaptosomal assay for site 1 using saxitoxin (Figure 3).

Using the additional natural brevetoxins we have developed specific displacement curves which correlate well with the potency of each individual purified toxin (Figure 4). We found it was very important to include the Emulphor EL-620 in all experimental tubes. The reason for this requirement, we surmise, is because of the differential lipid solubility of each of the natural brevetoxins and their tendency to form micelles.

In addition to developing displacement curves for the six toxins (n=2), we had sufficient toxin material for PbTx-1,-2,-3, and -7 to calculate K_is. These are shown in Figure 5.

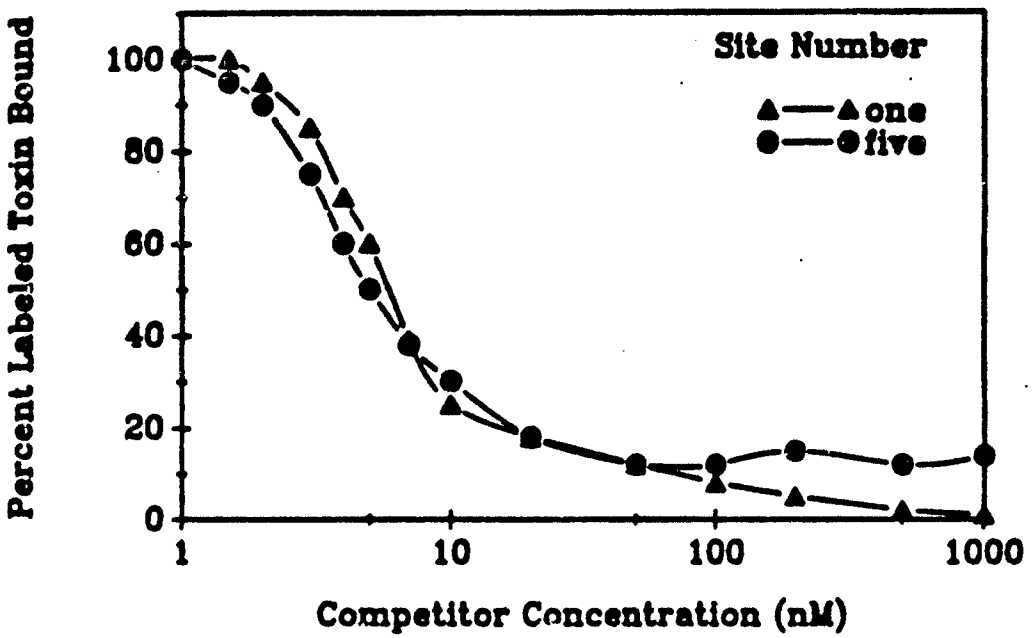


Figure 3. Specific Displacement of Labeled Toxins by Unlabeled Competitors, Sodium Channel Receptors. Site 1 probe is 10 nM tritiated saxitoxin; site 5 probe is 10 nM brevetoxin PbTx-3. Competitors are unlabeled saxitoxin and brevetoxin respectively.

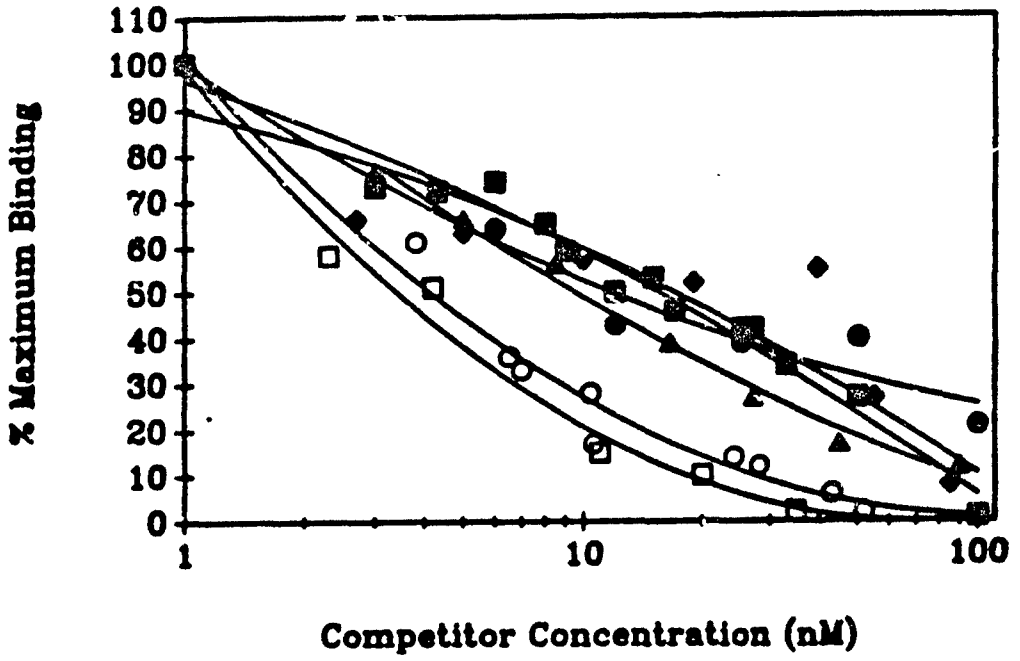


Figure 4. Effect of Brevetoxins on Tritiated PbTx-3 Binding to Rat Brain Synaptosomes. Incubations, in the presence of 50 ug synaptosomal protein and 16 nM tritiated PbTx-3 (10.15 Ci/mmole) with increasing amounts of unlabeled PbTx-1(□), PbTx-2 (■), PbTx-3 (●), PbTx-5 (▲), PbTx-6 (◆) or PbTx-7 (○), were for 1 hour at 4°C. Each point represents the mean of three triplicates. The ED_{50} in each curve is given in Table 3.

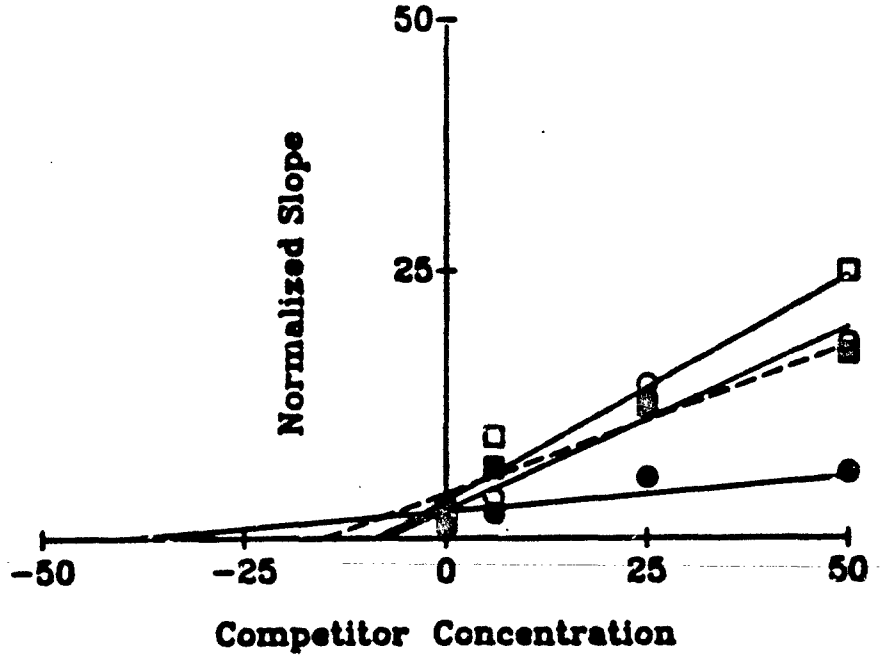


Figure 5. K_i Determinations for Brevetoxins. Rat brain synaptosomes were incubated with tritiated PbTx-3 concentrations of 2,5,10 and 20 nM, and unlabeled PbTx-1 (□), PbTx-2 (■), PbTx-3 (●), or PbTx-7 (○) at concentrations of 5, 25, 50, and 100 nM. Double reciprocal Lineweaver-Burke type plots were developed for each toxin and indicated competitive inhibition of binding. The slopes of the individual lines wer plotted against the competitor concentration in each case. K_is in each case are determined by the intersection of each line with the competitor axis, and is equal to $-K_i$.

We have collected sufficient competitive displacement information on PbTx-1,-2,-3,and-7 only, principally because they are abundant enough to gather sufficient toxin for the large number of individual tubes required for each experiment. Each line in Figure 5 is the result of at least 36 individual measurements. The K_is determined thus far approximate relative potencies of the materials; i.e. those with lower K_is are more potent. This work is still in progress however and additional replications are required before we prepare the results for juried journal publication. In addition, we plan to continue collecting PbTx-5 and -6 for use in similar experiments.

Competitive Displacement of Tritiated PbTx-3 by Natural Brevetoxins in Immunoassays. Radioimmunoassay displacement curves (Figure 6) indicate that the antibody recognizes and binds the toxins which possess the type of structure depicted on the left(type-1) of Figure 1 with much higher affinity than it does the toxins whose backbone is illustrated in Figure 1 on the right (type-2). This is not surprizing because the antibody was produced by immunization with BSA-linked PbTx-3, a type-1 toxin (18). Statistical analysis of ED_{50} values reveal that there are no statistical differences between the efficiencies with which PbTx-2, PbTx-3, and PbTx-5 displace tritiated PbTx-3 from the antibody-hapten complex (t-test, $p<0.1$). Analysis of 50% displacement values for PbTx-1 and PbTx-7 (both type-2) revealed no statistically-significant difference ($p<0.001$). With the exception of PbTx-6, a significant difference was consistently found, however, between the curves for the two toxin backbones. Type-1 toxins are approximately 10-fold more efficient than are type-2 toxins at displacing tritiated PbTx-3 from the binding site. The exceptional case, PbTx-6, is a 27,28 epoxide of a type-1 toxin. An epitope on the toxin molecule may involve the configuration around the 27,28 carbon unsaturation.

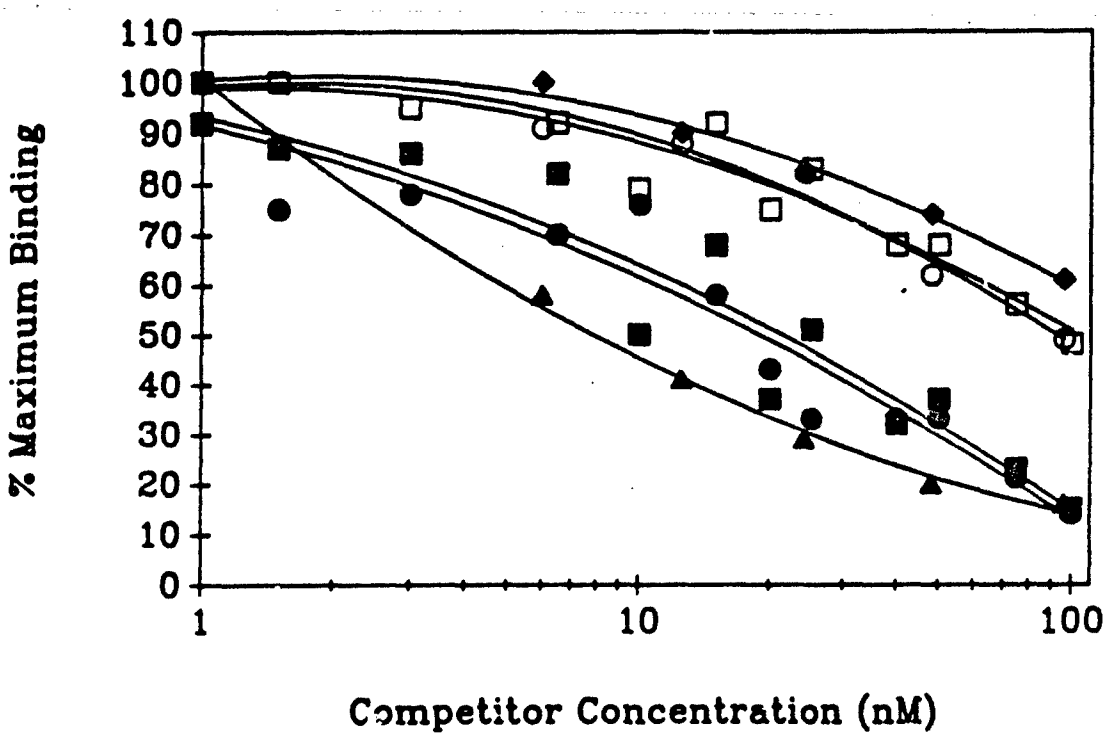


Figure 6. Effect of Brevetoxins on Tritiated PbTx-3 Binding to Antibodies. Incubations, in the presence of 0.75 mg antibody solution and 2 nM tritiated PbTx-3 (10.15 Ci/mmole) with increasing amounts of unlabeled PbTx-1 (□), PbTx-2 (■), PbTx-3 (●), PbTx-5 (▲), PbTx-6 (◆), or PbTx-7 (○), were for 24 hours at 4°C. Each point represents the mean of three triplicates. 50% effective dose concentrations are given in Table 3.

TABLE 3. CORRELATION OF POTENCY WITH RADIOIMMUNOASSAY AND SYNAPTOSOME ASSAYS

Toxin	Synaptosome ED_{50} (nM)	Synaptosome K_i (nM)	LD_{50} (nM)	Radioimmunoassay (nM)
PbTx-1	3.5	7.1	4.4	93.0
PbTx-7	4.1	8.9	4.9	92.0
PbTx-2	17.0	16.1	21.8	22.0
PbTx-3	12.0	37.0	10.9	20.0
PbTx-5	13.0	----	42.5	10.1
PbTx-6	32.0	----	35.0	112.0

ED_{50} are defined as the toxin conc at which 50% displacement of tritiated PbTx-3 from sodium channels or antibody occurs. LD_{50} are determined by incubation of Gambusia affinis with toxin in 20 mL seawater for 60 minutes. K_i are determined as described in the text.

In the synaptosomal assays (Figures 4-5), the displacement curves for brevetoxins possessing PbTx-2 type structural backbones show 50% displacement at 10-30 nM competitor concentrations. By comparison, PbTx-1 and PbTx-7 displace tritiated PbTx-3 at much lower concentrations; in both cases 50% displacement occurs at about 4 nM competitor concentrations. T-test analysis revealed no significant difference between the PbTx-2 backbone type toxin ED_{50}s ($p<0.01$), or between PbTx-1 and PbTx-7 ED_{50}s ($p<0.01$), but statistically significant differences were found between the curves generated by the two backbone classes.

The comparison of Gambusia affinis fish bioassays shown in Table 3 with the ED_{50}s and preliminary K_i data for each respective assay indicates that the two more potent ichthyotoxins, PbTx-1 and PbTx-7, are most efficient on a molar basis in displacing labeled PbTx-3 from its specific site of action in synaptosomes. There was no correlation between the potency of each toxin and the ability of each to displace tritiated PbTx-3 from the antibody.

The affinities of the toxins in the synaptosomal assays theoretically are based on differential structural considerations involved in the binding to the site on the sodium channels as well as lipid solubilities of each of the materials. It is noteworthy that PbTx-1 and -7, the two most hydrophobic toxins, are also the most potent and bind with tightest affinity to the site of action. The greater binding affinity of these two toxins may be a function of their flexibility across the D,E,F,G polyether portion (which confers about a 40° bending capability) as opposed to the rigid character of PbTx-2-like toxins. This added flexibility may allow these two toxins to conform better to the topography of the brevetoxins binding site.

It has been hypothesized that the brevetoxin binding site lies in an hydrophobic portion of the channel (8). This is consistent with the potency and hydrophobicity of PbTx-1

and -7. It is also noteworthy that oxidized PbTx-2 is impotent in fish bioassay, and that it lacks any capability to displace tritiated PbTx-3 from the binding site (9). It is our contention that the substituent character on C-42 in part determines solubility (and hence access to its synaptosomal site of action), and that the distal end of each backbone type carries the active portion of each toxin. Additional brevetoxin derivatives are being synthesized to test this hypothesis.

Thus, the affinities of the toxins in each assay are based on different structural considerations: in the case of immunoassay, affinity is based on all of the antigenic determinants on PbTx-3; in the synaptosome assay on the portion(s) of each toxin involved in binding to its active site in the channel. The inferior displacement ability of PbTx-1 and PbTx-7 in RIA can be attributed to the different structural backbone of these type-2 toxins. The larger 8 and 9 membered rings of these two toxins make the backbone less recognizable, perhaps because of added flexibility, or perhaps instead because of a less "stable" epitope. The only nearly identical parts of the respective two backbones are the H,I,J, and K rings of type-1 structures, and the G,H,I, and J tings of type-2 structures; the low displacement ability of PbTx-6 in RIA indicates that an epitope may lie near this region.

Enzyme-Linked Immunoassay Development. The basic assay under development follows a noncompetitive enzyme immunoassay sandwich technique (figure 7). Heterogeneous system assays (24,25) such as these may be performed as either competitive or noncompetitive types, and may be either enzyme-antibody labeled or enzyme-hapten(antigen) labeled. Thus, the greatest flexibility is gained employing such techniques, and many different variations may be developed to meet defined criteria.

In order for the proposed assay to work, toxin PbTx-2 had to be successfully bound to the microtitre plate wells. Unlike standard enzyme immunoassay procedures, where water-soluble IgG is adsorbed to the plastic plates, it was necessary to investigate the binding kinetics and equilibria of toxin binding. It is imperative that the solid phase should adsorb an adequate amount hapten in a reproducible manner, and that variability at this stage will affect the ultimate precision of the assay (26).

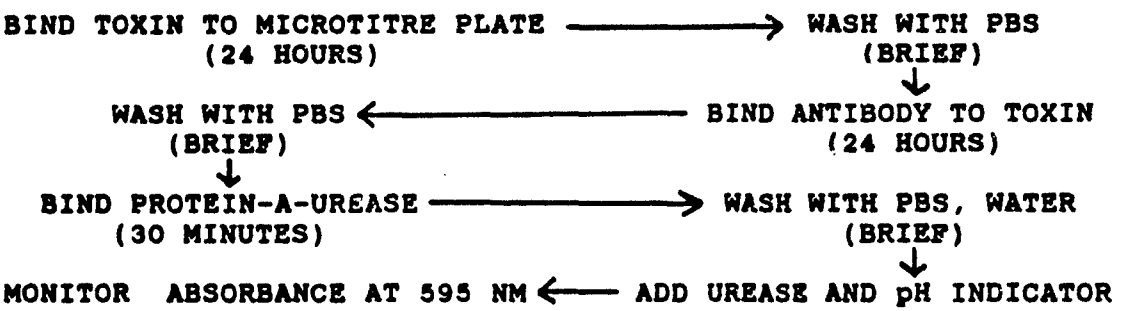


Figure 7. Flow Diagram of Enzyme-Linked Immunoassay Protocol.

Binding of PbTx-2 was evaluated in three media (figure 8): ethanol, a solvent in which the toxins are reasonably soluble; phosphate buffered saline, in order to promote partitioning onto the hydrophobic polystyrene surface; and carbonate buffer of pH 9.6, which is routinely used to bind IgG to plates. Following binding, complete Protein A-urease sandwich assays were carried out. The figure illustrates that PBS is the most suitable medium for toxin incubation. Incubation times shown in figure 8 represent the time course of the reaction, not the toxin pre-incubation time. This figure also serves to illustrate the linearity of the assay with respect to reaction time, illustrating the lack of end-product inhibition of the urease system.

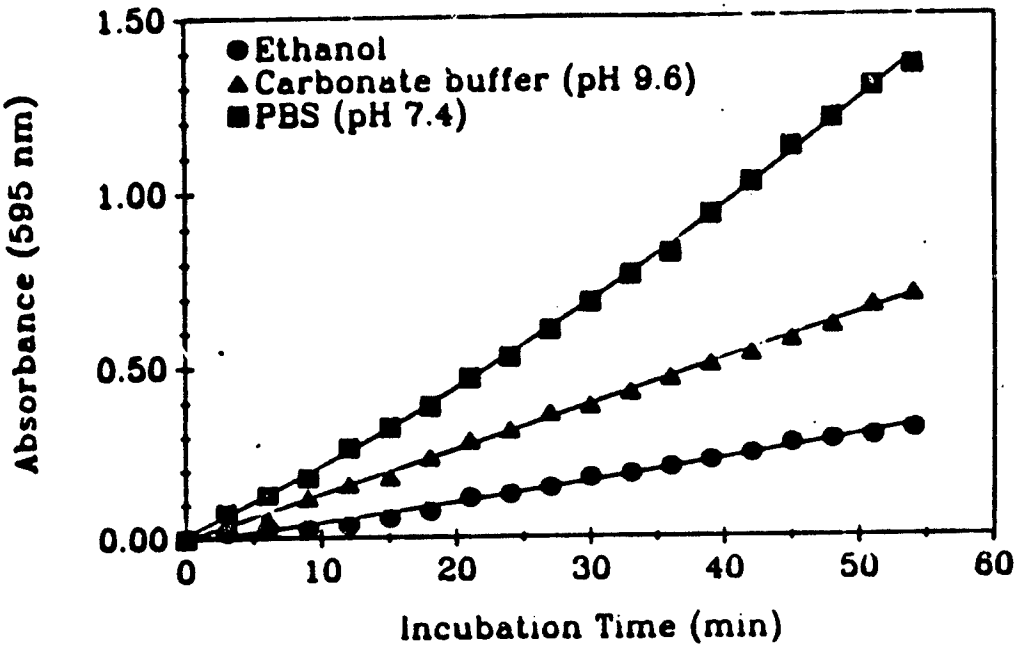


Figure 8. Brevetoxin Enzyme-Linked Immunosorbant Assay: PbTx-2-Antitoxin adsorbed to Microtitre Plates; Protein A-Urease detection of toxin-antitoxin complex. One ug toxin per plate, pre-incubated for 24 hours, and then subject to assay as described above. Absorbance at 595 nm is used as a criteria of pH change (bromocresol purple) due to cleavage of ammonium ion from urea substrate. Change in absorbance with time in the incubation indicates linearity of the assay over 60 minutes.

The stability of the toxin-antitoxin adsorbed on the microtitre plate, when stored in a dry atmosphere at room temperature, indicates the probable long shelf-life of the reagents (figure 9). The stability curve has been carried out for 2 months now, with no loss in activity.

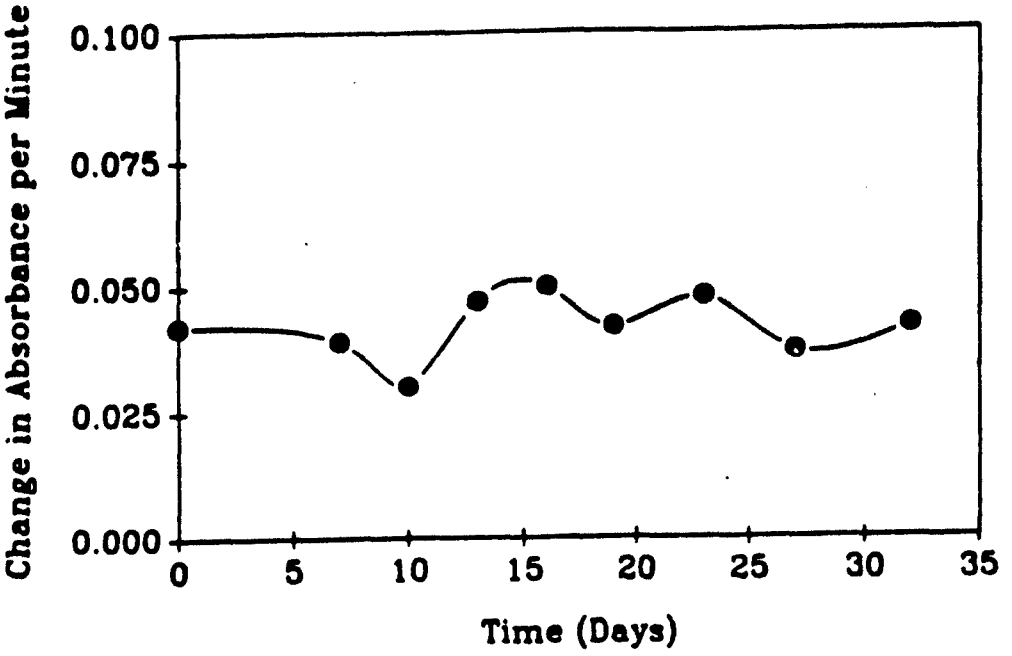


Figure 9. Stability of the PbTx-2-Antitoxin Complex Adsorbed to Microtitre Plates. Two 8x12 96 well microtitre plate were presorbed with toxin and antitoxin, and weres then stored in a dessicator at room temperature of 2 months, assaying at timed intervals as shown above. The protein A-urease assay was carried, and changes in absorbance per minute was calculated for each assay. Points are an average of 12 wells in the microtitre plate array.

VI. Conclusions

Laboratory cultures of Ptychodiscus brevis produce at least six different polyether toxins, derived from one of two structural classes. During logarithmic phase, two of the toxins predominate and both are alpha-beta unsaturated aldehydes based on different structural backbones. Upon reaching stationary phase, four additional toxins appear, three based on the structural backbone present in PbTx-2, and an additonal single toxin based on the structural backbone present in PbTx-1. The additional toxins have been hypothesized to arise from classical detoxification reactions carried out on the two alpha-beta unsaturated aldehyde toxins.

Each toxin displaces tritiated PbTx-3 from its specific site of action in synaptosomes; all ED_{50}s occur in the 3-30 nM concentration ranges with the more lipid soluble toxins being more efficient at displacing labeled toxin. The ability to displace radioactive toxin from the binding site correlates in a positive fashion with fish potency. Preliminary K_i calculations for four of the toxins indicate a similar positive correlation.

Brevetoxins have been compared in their ability to displace tritiated PbTx-3 from specific antibodies raised in a goat. In radioimmunoassay, the ability to displace appeared to be based soley on each toxin's structural similarity to PbTx-3, the hapten against which the antibodies were raised.

Enzyme-linked immunoassays employing polystyrene-adsorbed PbTx-2, antitoxin, and a Protein A urease detection system appear promising. Shelf-life appears good, detection using a microtitre plate reader is convenient, reproducible, linear, and rapid. Evaluation of serum titres should proceed smoothly with this assay.

VII. Recommendations

(1) Complete examination of tritiated PbTx-3 binding under potassium ion depolarizing conditions. We have thus far been unsuccessful in detecting any difference between normal polarized state and under conditions of membrane depolarization;

(2) explore specific binding of tritium labeled brevetoxin PbTx-3 to detergent-solubilized binding component from sodium channel to develop a soluble assay akin to RIA;

(3) examine the feasibility of producing a urease-linked brevetoxin probe for (2) above and for development of an ELISA using specific brevetoxin antibodies;

(4) continue refinement of the Protein A-urease ELISA;

(5) examine competitive displacement using other brevetoxins. This applies to (1)-(4);

(6) begin evaluation of brevetoxin quantitation in biological fluids using the RIA currently available, and utilizing (1)-(4) as they are refined.

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